NAVAL MEDICAL RESEARCH UNIT SAN ANTONIO

SILVER NANOPARTICLE STORAGE STABILITY IN AQUEOUS AND BIOLOGICAL MEDIA

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CRANIOFACIAL HEALTH AND RESTORATIVE MEDICINE

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DECLARATION OF INTEREST

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ABBREVIATIONS

$AgNO_3$	Silver Nitrate
AgNP	Silver Nanoparticle
au	Arbitrary Units
Da	Dalton
$NaBH_4$	Sodium Borohydride
$Na_3C_6H_5O_7 \cdot 2H_2O$	Tribasic Sodium Citrate dihydrate
PVP	Poly(vinylpyrrolidone)
FWHM	Full Width Half Maximum
H_2O_2	Hydrogen Peroxide
Media	Eagle's Minimum Essential Medium without horse serum
Media+S	Eagle's Minimum Essential Medium supplemented with horse serum
MDR	Multi-Drug Resistant
PBS	Phosphate Buffered Saline
TEM	Transmission Electron Microscopy
TSB	Tryptic Soy Broth
UV-vis	Ultraviolet-visible absorption spectroscopy

EXECUTIVE SUMMARY

Background: A high risk for multidrug-resistant infections was observed in wounds suffered during the conflicts in Iraq and Afghanistan. Novel antimicrobial agents are needed to combat the rise in multidrug-resistant infections. Silver has antimicrobial effects against a broad spectrum of bacteria and has been successfully incorporated into wound treatments to reduce infections. Dressings and implant coatings are being developed which integrate silver nanoparticles (AgNPs) for their long-term antimicrobial properties. Silver ions are known to slowly leech from the surface of nanoparticles, which destabilizes the particles. Additionally the medium components that interact with the nanoparticles play a significant role in the destabilization of the nanoparticle over time. Although this interaction plays an important role in the long-term use and storage of AgNPs, there are few reports describing the stability and storage of the nanoparticles. Therefore, it is crucial to fully understand the effects of storage on silver nanoparticles. The objective of this study was to evaluate the stability of silver nanoparticles stored for 14 days in different aqueous and biological media.

Methods: Silver nanoparticles were incubated in water, cell culture media, and tryptic soy broth (TSB) over 14 days at a concentration of 30 µg/ml. The conditions were conducted at different temperatures, 4°C, room temperature (22-25°C), and 37°C. At 7 and 14 days, nanoparticle stability was monitored using Ultraviolet-visible spectroscopy and transmission electron microscopy.

Results: Silver nanoparticles were stable for 14 days in de-ionized water without significant changes to size or morphology. However, silver nanoparticles showed marked aggregation after 14 days in cell culture media or TSB. Refrigeration at 4°C and protection from light were necessary for storage stability.

Conclusions: Degradation or aggregation of the silver nanoparticles over time may influence their impact on cell toxicity, antimicrobial effects, and bacterial attachment. This study establishes the conditions for silver nanoparticle stability from the point of synthesis to the point of testing. The recommended conditions of water storage at 4°C protected from light should be used when designing and conducting future studies. Standardization of storage conditions for silver nanoparticles could decrease undesirable changes in size and morphology, and increase the validity of research.

INTRODUCTION

Preventing and treating infections in severely wounded or burned warfighters presents major challenges. Warfighters wounded in austere field environments increasingly encounter multi-drug resistant (MDR) pathogens[1-3]. Treatment of MDR infections requires broad-spectrum antibiotics, which are more toxic, less effective, and more expensive than those specific to one bacterial strain[4]. Furthermore, MDR pathogens increase recovery time and extend the hospital stay. Warfighters receiving surgical implants are also at increased risk of infection. For example, post-operative infection rates associated with orthopedic cranial procedures in the military are estimated to be 5 to 8%[5]. With a cost of $50,000 per surgical intervention, the total cost of surgical interventions due to infections could reach $120 million[5]. A general therapy effective against MDR flora that could be incorporated into topical treatment of war wounds, as well as onto implant coatings, is a high priority.

Nanosilver has been manufactured in the United States since 1897 and used for medical purposes since the early part of the twentieth century[6]. The therapeutic effect of nanosilver originates from the free silver ion, which is known to be bactericidal for over 150 species of bacteria, including Methicillin-resistant *Staphylococcus aureu*s and MDR *Acinetobacter*, *Klebsiella* and *Pseudomonas species*. Burn therapies have utilized elemental silver treatments in the form of dilute silver nitrate solution since 1965 and in silver-sulfadiazene since 1968[7-9]. Ten commercially available silver-nylon dressings have been approved by the US Food and Drug Administration, and Silverlon® has been extensively used to treat burn patients in military hospitals and during evacuation from combat since 2003[10].

Silver nanoparticles (AgNPs) are being explored for their potential use in antimicrobial dressings. There are a number of concerns that might impact the efficacy of AgNPs over a long period of time. Silver ions slowly elute from the surface of AgNPs and the antimicrobial effectiveness of AgNPs is highly dependent upon the delivery of silver ions at bactericidal concentrations[11]. Few studies have been done to explore the stability of AgNPs in different biological and aqueous media, as well as the effects of temperature. The current study attempts to improve the understanding of the storage conditions that might impact the efficacy of AgNPs for the design of future toxicity and bacterial studies. The objective of this study was to identify storage limits in different media, and to develop a standardized method for the long-term storage of AgNPs.

MATERIALS AND METHODS

Materials

Silver nitrate ($AgNO_3$, >99%), tribasic sodium citrate dihydrate ($C_6H_5O_7Na_3.2H_2O$, >99%), sodium borohydride ($NaBH_4$, 99%), hydrogen peroxide (H_2O_2. 30%), Poly(vinylpyrrolidone) (PVP; Mw ~56,000) were purchased from Sigma–Aldrich (St. Louis, MO). Ultrapure DNase/RNase-free distilled water was used for the preparation of solution and as the aqueous storage medium (Invitrogen, Grand Island, NY). Eagle's Minimum Essential Medium (EMEM) and horse serum were purchased from ATCC (Manassas, VA). Tryptic soy broth was purchased from BD (Franklin Lakes, NJ).

Preparation of AgNPs

Silver nanoparticles were synthesized using a procedure adapted from the thermal method of Métraux and Mirkin[13]. All stock solutions used for the AgNP fabrication were prepared immediately prior to their addition. First, 25 ml of aqueous solution of $AgNO_3$ (0.2 mM) was prepared in a 50 ml glass beaker and used as the precursor solution for the fabrication of AgNPs. Under continuous stirring at 600 RPM on a Lab Companion Multi-Channel stir plate (JeioTech, Seoul, Korea), sodium citrate (30mM, 0.75 ml) was added. This was followed by addition of the stabilizing agent PVP of molecular weight 56,000 Daltons. H_2O_2 (30%, 30 µL) and sodium borohydride (100mM, 150uL) were rapidly injected, generating a solution that was initially pale yellow in color. The resultant solution was stirred, in the dark, for an additional hour for the reaction to proceed to completion.

Storage Stability and Characterization of Silver Nanoparticles

The stability of AgNPs was evaluated in water, TSB, EMEM with 10% horse serum (Media+S), EMEM only (Media), phosphate buffer saline (PBS), and 5% glucose over a 14 day storage period. After synthesis, AgNPs were first purified by ultracentrifugation using an Optima® L-80XP Centrifuge (Beckman Coulter, Brea, CA) to remove unreacted reagents and excess silver ions (24,000 RPM, 2 hours, 4°C). The supernatant was removed using a 5 ml syringe with an 18 gauge needle (BD Biosciences, Franklin Lakes, NJ). The remaining pellet was reconstituted in 1 ml of storage medium and then aliquots were further diluted to a final concentration of 30 µg/ml. Atomic absorption spectroscopy was used to confirm the final

concentration of 30 μg/ml. Reconstituted AgNPs were stored at 4°C or 37°C in the dark, unless otherwise specified, for 14 days. The AgNP suspensions were analyzed at 0, 7, and 14 days by Ultraviolet-visible (UV-vis) spectroscopy using a Synergy HT plate reader (BioTek Instruments, Winooski, VT) with 75μl suspension per well in clear polystyrene 96 well plates (BD Falcon, Franklin Lakes, NJ). Silver nanoparticle morphology was evaluated at 0, 7, and 14 days by transmission electron microscopy (JEOL100CX, Tokyo, JPN). Transmission electron microscopy (TEM) samples were prepared by depositing 5 μl of sample onto a 300 mesh carbon grid (Electron Microscopy Sciences, Hatfield, PA), lightly blotting the grid, and then drying the sample for 5 minutes. Thirty TEM images were collected and the size distributions of at least 300 particles per sample were generated from the images using ImageJ (NIH, Bethesda, MD) software.

RESULTS

Reconstitution of AgNPs in Biological Media

Reconstitution of AgNPs at 30μg/ml in water resulted in a UV-vis peak centered at 480 nm, while re-suspension of AgNPs in TSB and Media+S resulted in a broader UV-vis peak centered at 585 and 570 nm, respectively (Fig. 1A). In contrast, re-suspension into media in the absence of horse serum led to a shift of the UV-vis spectra towards shorter wavelengths, with a peak maxima observed at 430 nm (Fig. 1A). The blue shift of the particle absorption spectra for AgNPs in media corresponded to more than a 50% decrease in particle diameter. AgNPs had a mean particle diameter of 12 ± 4 nm in water, 16 ± 6 nm in TSB, 14 ± 5 nm in Media+S, and 5 ± 3 nm in media (Fig. 1B). Additionally, AgNPs were reconstituted in glucose and PBS to identify the main components responsible for the shift in absorption in each of the mediums. Glucose resulted in a mild shift of the peak from 480 nm to 510 nm, while PBS resulted in a significant shift from 480 nm to 420 nm (Fig. 1A insert). The blue shift of the absorption spectra for PBS was similar to that observed for media, suggesting that inorganic salts destabilize the AgNPs. The absence of the blue shift in TSB and Media+S was most likely due to the presence of serum components which protect AgNPs from the destabilizing salts.

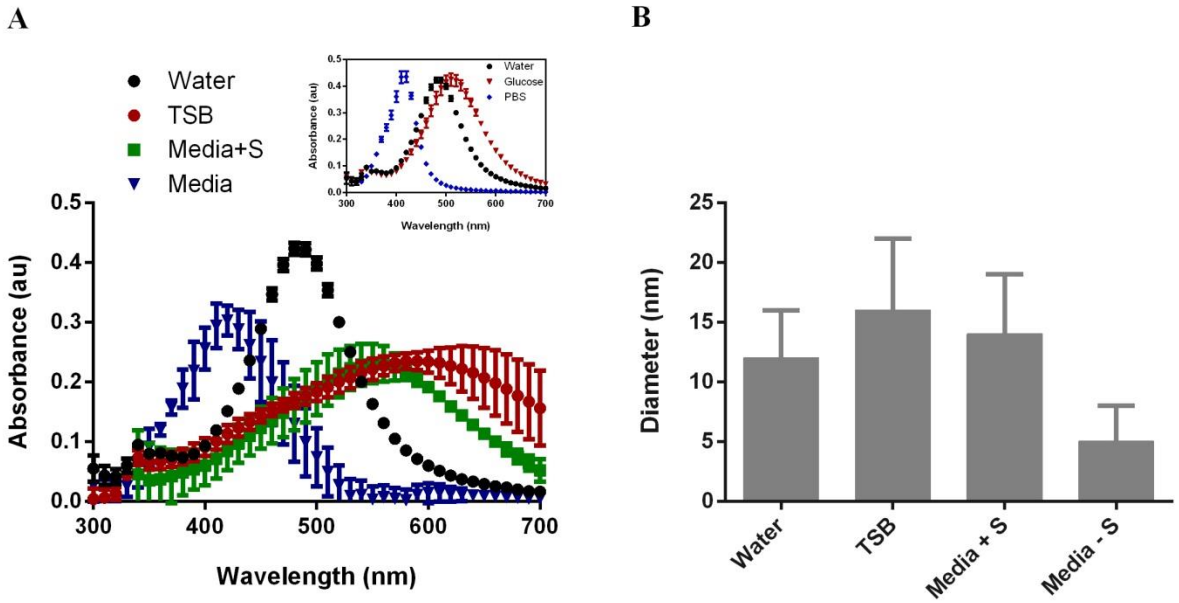

Figure 1: Characterization of AgNPs immediately following reconstitution in water and biological medium. A) UV/vis spectra of AgNPs immediately following reconstitution. Insert: Reconstitution in PBS and 5% glucose. Silver nanoparticles reconstituted in water and glucose showed a strong peak centered at 480 nm. Silver nanoparticles reconstituted in TSB and Media+S exhibited a broader peak shifted towards longer wavelengths. Reconstitution in media only lead to a shift towards shorter wavelengths similar to that observed upon reconstitution in PBS. The difference in spectra between the Media+S and media only suggest that the presence of serum proteins form a protective coating on the AgNPs which prevent salts from destabilizing the AgNPs. B) Mean AgNP diameter obtained from TEM images. Silver nanoparticle diameters were 12 ± 4 nm in water, 16 ± 6 nm in TSB, and 14 ± 5 nm in Media+S. Silver nanoparticle diameter of 5 ± 3 nm was significantly smaller when reconstituted in media only.

Stability of Silver Nanoparticles in Biological Culture Medium

The UV-vis absorption peaks of AgNPs in TSB or Media+S over time at both 4°C and 37°C are shown in Figure 2. The absorbance decreased from 0.24 ± 0.02 to 0.17 ± 0.03, while the peak shifted from 585 to 570 nm for TSB (Fig. 2A). The absorbance decreased from 0.23 ± 0.02 to 0.21 ± 0.03, while the peak shifted from 570 to 545 nm for Media+S (Fig. 2C). Both mediums resulted in a slight decrease in absorbance at the peak and a shift towards shorter wavelengths after 7 days of storage at 4°C. In contrast, storage over the same time period at 37°C resulted in a significant decrease in absorbance from 0.25 ± 0.05 to 0.14 ± 0.02 for Media+S, and almost complete disappearance of the peak for TSB (Fig. 2B and 2D). The UV-vis absorption peaks indicated AgNP instability during long-term storage in biological medium, and this instability is accelerated at 37°C.

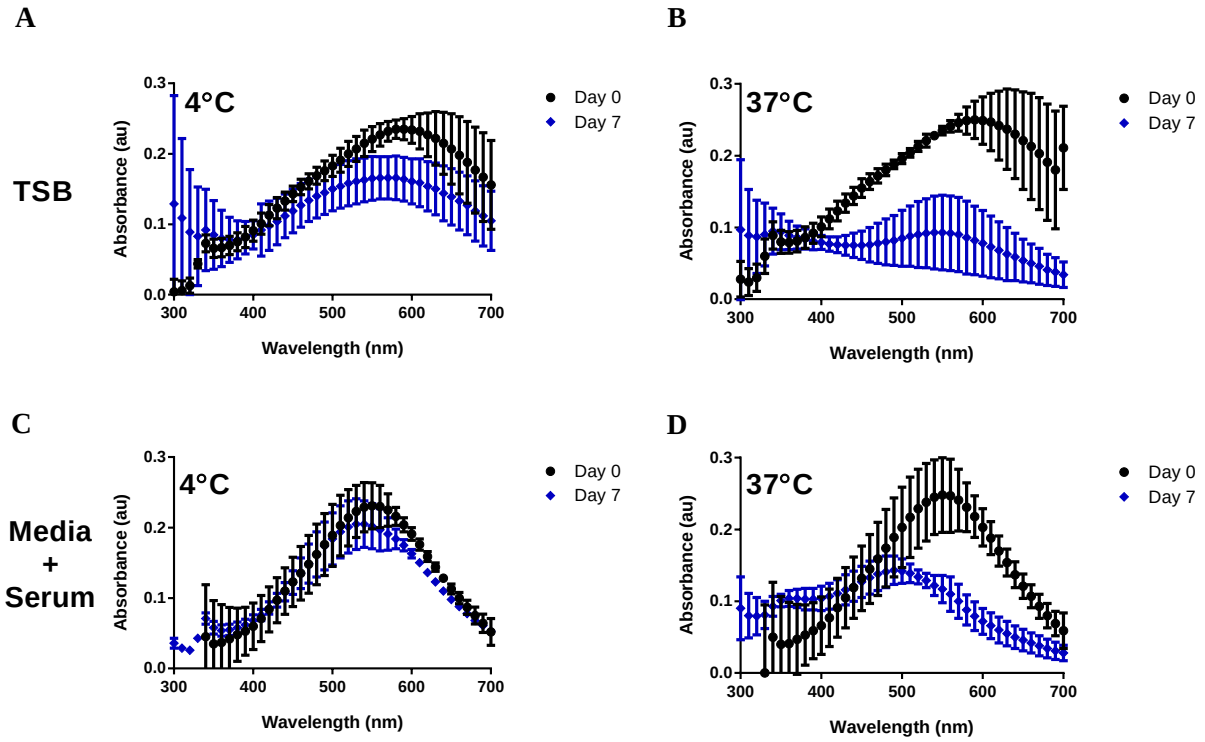

Figure 2: UV/vis spectra of AgNPs stored for 7 days in A) TSB at 4°C, B) TSB at 37°C, C) Media+S at 4°C, and D) Media+S at 37°C. The absorbance at the peak decreases over 7 days in TSB and Media+S. The decrease in absorbance after 7 days is greater when AgNP are stored at 37°C, indicating that particle instability is accelerated at 37°C.

Transmission electron microscopy images confirmed the gradual loss of AgNP stability when stored at 4°C in biologically relevant media. After 14 days, AgNPs in TSB and Media+S showed aggregation that was visible under TEM with particles fused into large clusters of greater than 100 nm (Fig. 3). Figure 4 shows the final time point at which AgNPs remained stable in different medium. After 7 days, the particles remained dispersed within TSB and Media+S. Histograms of the size distribution suggested a slight shift towards larger particles over time, although the mean diameter remained constant at 16 ± 6 nm for TSB and only increased from 14 ± 5 to 15 ± 6 nm for Media+S. Silver nanoparticles stored within TSB and Media+S were therefore stable over 7 days, but aggregate between 7 to 14 days of storage. In the absence of serum, AgNPs were unstable upon reconstitution in media and had a mean diameter of 5 ± 3 nm. These particles aggregated during storage into larger particles which then clustered into large macroscopic aggregates and settled out of solution within 3 days at 4°C.

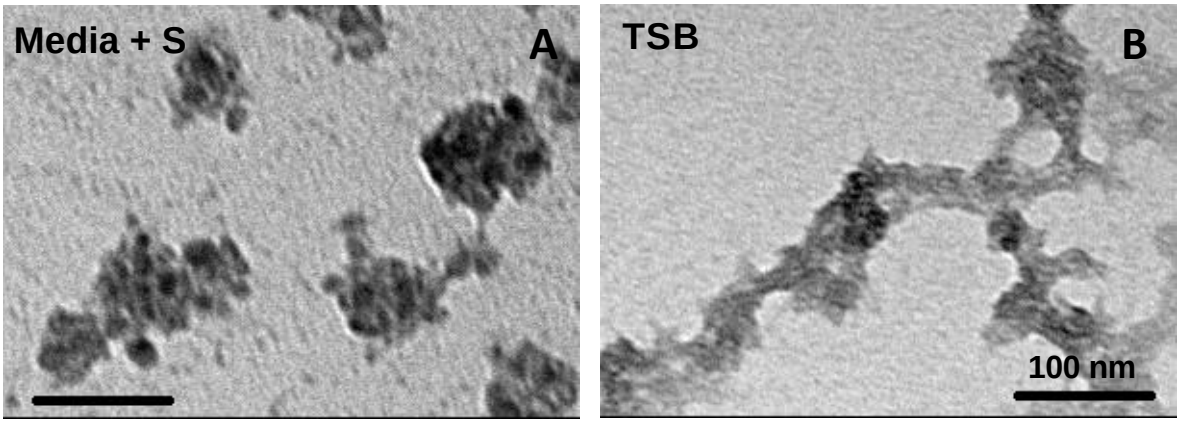

Figure 3: TEM images of AgNPs after 14 days of storage at 4°C in biological medium of Media+S (A) and TSB (B). Silver nanoparticles have aggregated during storage and are not stable for 14 days in these medium.

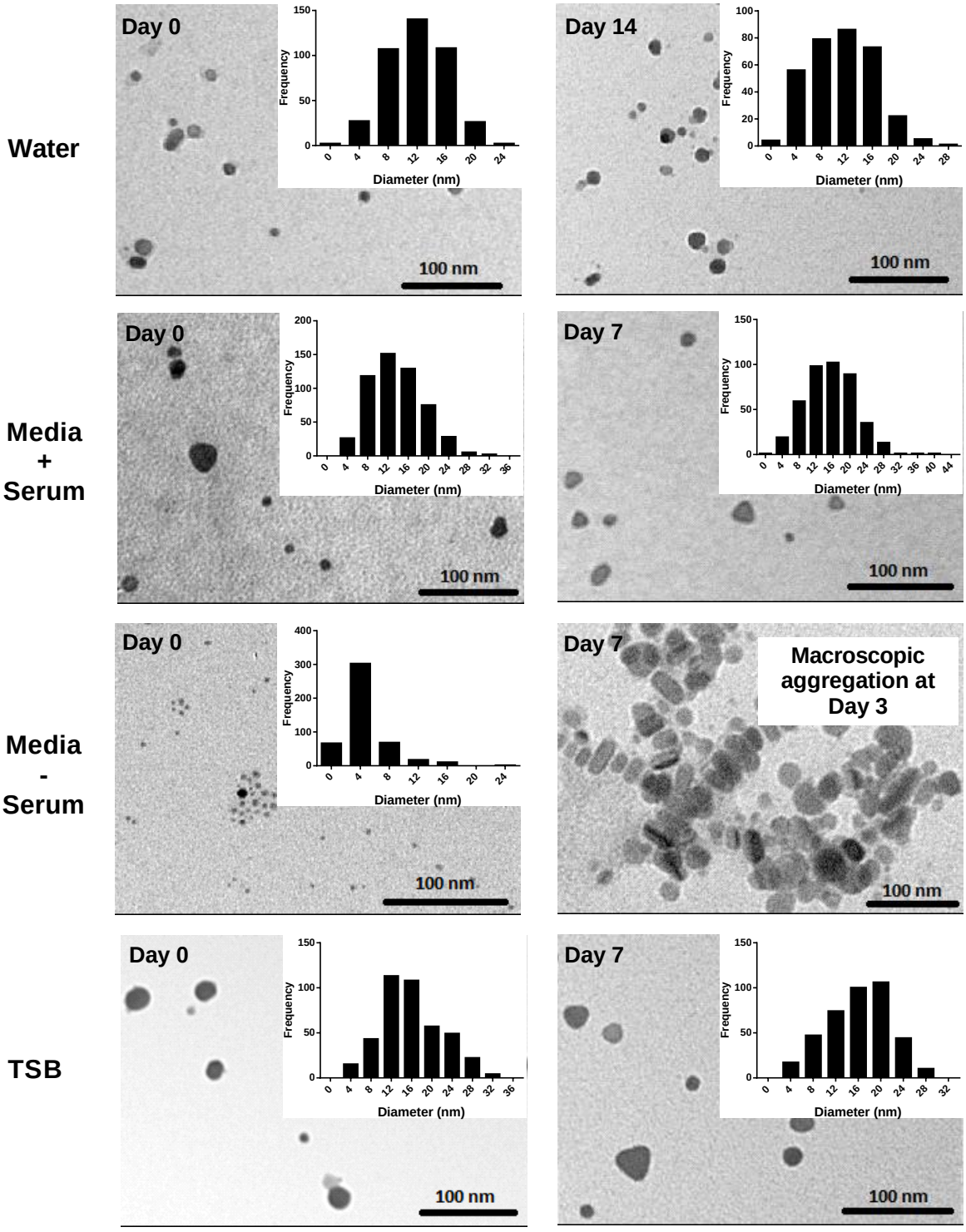

Figure 4: TEM images showing the final time point at which AgNPs remained well dispersed. Silver nanoparticles remain well dispersed after 14 days in water with a particle size of 12 ± 4 nm. Fourteen day TEM images are not shown for Media and TSB, as the AgNPs were destabilized after 7 days in Media+S and TSB. Images of AgNPs in Media+S and TSB at 14 days showed particle aggregates. Silver nanoparticles were destabilized into 5 ± 3 nm particles immediately upon reconstitution in media only, which cluster into aggregates visible by the naked eye within 3 days. Inserts: Size histograms obtained from the TEM images.

Stability of Silver Nanoparticles in Water

In contrast to storage in biological culture medium, AgNPs stored in water were well-dispersed at both 0 and 14 days and the particle diameter remained constant at 12 ± 4 at 0 days and 11 ± 5 nm at 14 days. These results indicate that AgNPs remain stable over 14 days in water at 4°C. Storage at room temperature, exposure to light, and storage at high concentration were evaluated to further refine the storage parameters under which AgNPs remain stable. The UV-vis spectra show that the AgNP peak remained at 480 ± 10 nm when stored for 14 days at both 4°C and at room temperature of approximately 22°C (Fig. 5A and B). Significant broadening of the peak due to particle aggregation was not observed, as the full width half maximum (FWHM) only changed from 115 nm to 110 nm for 4°C storage and from 115 nm to 125 nm for room temperature storage. Exposure to light impacted the storage stability of AgNPs, as this batch of AgNPs had an initial peak at 540 nm which shifted to 460 nm when stored under ambient lighting, versus a shift from 540 nm to 520 nm when the sample was protected from light by an aluminum foil wrap (Fig. 5C). Silver nanoparticle concentration during storage also had no impact on stability, as AgNPs stored within a concentrated pellet at approximately 1000 μg/ml and then diluted to 30 μg/ml after 14 days had a UV-vis spectra equivalent to AgNPs stored at 30 μg/ml for 14 days (Fig. 5D). These studies indicated that AgNPs must be stored within water and protected from light for long-term stability.

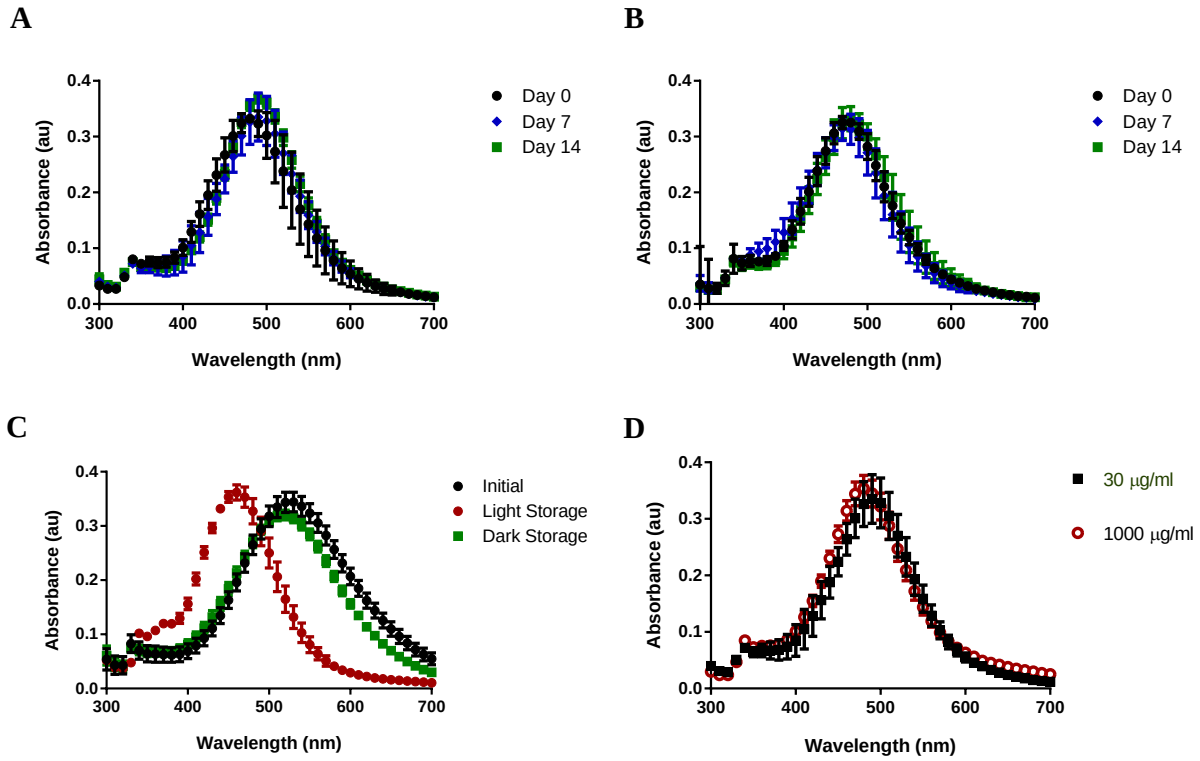

Figure 5: Silver nanoparticles stored in water for 14 days. A) Silver nanoparticle UV/vis spectra remained at 480±10 nm during storage at 4°C. B) Silver nanoparticle UV/vis spectra remained at 480±10 nm during storage at room temperature protected from light. C) This batch of AgNPs had an initial UV/vis spectra centered at 540 nm, which underwent a small shift to 520 nm when stored at room temperature protected from light but underwent a larger shift to 460 nm when stored at room temperature exposed to ambient light. D) Silver nanoparticle UV/vis spectra were equivalent for AgNPs stored at 4ºCat concentrations of 30 µg/ml and 1000 µg/ml. All samples were analyzed at a concentration of 30 µg/ml.

DISCUSSION

A key objective of this study was to determine the storage stability limits of AgNPs in biological medium. These limits provide information necessary for designing cell toxicity, antimicrobial, and bacterial attachment studies which use AgNPs. Cell culture studies are performed within cell culture media, typically supplemented with serum, while bacterial studies are performed within TSB. Reconstitution of AgNPs in the appropriate biological medium prior to the experiment would simplify preparation on the day of the experiment. The results suggest that AgNPs can be prepared up to 7 days in advance of a study, assuming they are stored refrigerated at 4°C and protected from light. During cell and bacteria testing performed at 37°C, a gradual particle aggregation can be expected to occur, which may complicate long-term studies. During preliminary cell and bacteria studies with AgNPs incubated for only 1 and 2 days, particle aggregation was not expected to significantly interfere with the results. More

detailed information on the rate of AgNP aggregation in biological medium at 37°C, the rate and method of association of AgNPs with cell and bacteria membranes, and the impact of AgNP size on membrane interactions would be needed to confirm this hypothesis.

Results demonstrated that the stability of the AgNPs was also dependent on the components within the medium used for reconstitution. Immediately upon reconstitution in biological medium, the absorption peak broadens and was shifted towards longer wavelengths which are indicative of gradual aggregation of the AgNPs, or shifted towards shorter wavelengths which are indicative of destabilization of the AgNPs. Biological media contain a mixture of inorganic salts, amino acids, vitamins, and glucose (Appendix). The proteins present within TSB and media supplemented the medium with 10% horse serum were crucial to long-term structural integrity of AgNPs. Silver nanoparticles were stabilized within protein-containing biological medium for at least 7 days, whereas in the absence of protein, TEM images showed that they rapidly oxidized into smaller (< 5 nm) particles. It can be hypothesized that the proteins associated with the surface of the AgNPs prevented the inorganic salts from interacting with and destabilizing the AgNPs.

Proteins also appeared to slow the aggregation of AgNPs that occurred over extended storage. Cell culture media and TSB both contain inorganic salts, which are known to induce nanoparticle aggregation. At the level found in biological medium, 0.91% for media and 0.75% for TSB, rapid aggregation of nanoparticles was expected in the absence of a protective surface coating. The AgNPs were synthesized in the presence of the polymer PVP, and this formed a polymeric coating which was necessary for the formation of the 10 to 20 nm diameter AgNPs, as well as for their subsequent stability[12]. This polymeric coating was insufficient to fully stabilize the nanoparticles during storage; the AgNPs visibly aggregated and fell out of solution within 3 days in media. In contrast, TEM images indicated that the AgNPs remained well-dispersed after 7 days in media that was supplemented with proteins through the addition of horse serum. The AgNPs continued to gradually aggregate despite the polymer and protein coating, as indicated by the progressive broadening of the absorbance peak and the appearance of visible nanoparticle clusters in TEM images after 14 days of storage in biological medium. However, proteins appear to form a crucial secondary barrier that assists with long-term stability in the presence of complex medium.

It was determined that water is the preferred medium for long-term storage of AgNPs. Both absorption spectroscopy and TEM analyses indicate that AgNPs are stable for 14 days in water. The peak absorption remains constant around 480 nm, and the particles remain well-dispersed with a diameter of 11 ± 5 nm. Also, AgNPs are light-sensitive, such that samples must be wrapped in foil or stored within an amber container. Additionally, refrigeration at 4°C is recommended for long-term storage. This is suggested as a precaution to maximize stability, although the results indicated AgNP stability can be maintained at room temperature over 14 days. Although the current work presented here did not explore frozen storage, it can be anticipated that a freezing and thawing process will interfere with stability and might necessitate additional stabilizing agents. Finally, long term storage of the AgNPs at high concentration is discouraged. Storage concentration of the AgNPs on stability was evaluated by comparing storage at 30 μg/ml and 1000 μg/ml, corresponding to the upper concentration used for biological studies and maximum concentration achieved through ultracentrifuge processing, respectively. Upon reconstitution at 30μg/ml after 14 days of storage at 1000 μg/ml, the more concentrated AgNP had an absorption spectrum that was equivalent to that of the AgNPs stored at 30 μg/ml for the entire 14 days. Thus, highly concentrated samples have a greater potential for aggregation due to the relatively closer proximity of the nanoparticles, and are thus recommended only for short periods of time.

In conclusion, the current work identified the storage stability conditions for AgNPs in aqueous and biological media. Silver nanoparticles are stable for up to 14 days in water when refrigerated and protected from light. Gradual aggregation occurs in biological medium, and AgNP suspensions should be used for testing within 7 days of preparation. Future work could further explore the interaction of AgNPs with cells and bacteria to better understand toxicity.

MILITARY SIGNIFICANCE

The pathogens encountered in military practice are increasingly multi-drug resistant, and call for broad-spectrum antibiotics and antimicrobial agents. Silver ions are bactericidal against over 150 species of bacteria as well as MDR strains. Silver sulfadiazine cream and silver-nylon dressings are currently used to prevent infection in severe burns and wounds. Dressings with a slow release of silver ions require less frequent changes, which reduces the personnel time and material requirements associated with treatment in combat environments and during evacuation.

Silver nanoparticle-containing dressings have the potential to improve wound and burn treatment for both Wounded Warriors wounded in-theatre as well as civilian populations.

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APPENDIX

Table 1. Formulation for EMEM ATCC 30-2003 (g/L)

Inorganic Salts	**9.1376**		**Amino Acids**	**0.9542**
CaCl2 (anhydrous)	0.2000		L-Alanine	0.0089
MgSo4 (anhydrous)	0.0977		L-Arginine HCl	0.1264
KCl	0.4000		L-Asparagine H2O	0.0150
NaHCO3	1.5000		L-Aspartic Acid	0.0133
NaCl	6.8000		L-Cystine 2HCl	0.0312
NaH2PO4 H20	0.1400		L-Glutamic Acid	0.0147
			L-Glutamine	0.292
D-Glucose	**1.0000**		Glycine	0.0075
			L-Histidine HCl H2O	0.0419
Vitamins	**0.0081**		L-Isoleucine	0.0525
Choline Chloride	0.0010		L-Leucine	0.0525
Folic Acid	0.0010		L-Lysine HCl	0.0725
Myo-Inositol	0.0020		L-Methionine	0.0150
Nicotinamide	0.0010		L-Phenylalanine	0.0325
D-Pantothenic Acid	0.0010		L-Proline	0.0115
Pyridoxine HCl	0.0010		L-Serine	0.0105
Riboflavin	0.0001		L-Threonine	0.0476
Thiamine HCl	0.0010		L-Tryptophan	0.0100
Phenol Red, Sodium salt	0.0100		L-Tyrosine 2Na 2H2O	0.0519
Sodium Pyruvate	0.1100		L-Valine	0.0468

Table 2. Formulation for TSB (g/L)

BactoTM Tryptone	17.0
Bacto Soytone	3.0
Glucose	2.5
Sodium Chloride	5.0
Dipotassium Hydrogen Phosphate	2.5

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14. ABSTRACT

Silver has antimicrobial effects against a broad spectrum of bacteria and has been successfully incorporated into wound treatments to reduce infections. Dressings and implant coatings are being developed which integrate silver nanoparticles (AgNPs) for their long-term antimicrobial properties. The medium components that interact with the nanoparticles play a significant role in the destabilization of the nanoparticle over time. Although this interaction plays an important role in the long-term use and storage of AgNPs, there are few reports describing the stability and storage of the nanoparticles. The objective of this technical report was to evaluate the stability of silver nanoparticles stored for 14 days in different aqueous and biological media. Silver nanoparticles were stable for 14 days in de-ionized water without significant changes to size or morphology. However, silver nanoparticles showed marked aggregation after 14 days in cell culture media or tryptic soy broth. Refrigeration at 4°C and protection from light were necessary for storage stability. Degradation or aggregation of the silver nanoparticles over time may influence their impact on cell toxicity, antimicrobial effects, and bacterial attachment. This study establishes the conditions for silver nanoparticle stability from the point of synthesis to the point of testing. The recommended conditions of water storage at 4°C protected from light should be used when designing and conducting future studies. Standardization of storage conditions for silver nanoparticles could decrease undesirable changes in size and morphology, and increase the validity of research.

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MDR, Silver Nanoparticles, AgNPs, Storage

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